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The Effect of Amygdalin on the Process of Regeneration in the Adult Newt, *Notophthalmus Viridescens viridescens* (rafinesque)

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THE EFFECT OF AMYGDALIN ON THE PROCESS OF
REGENERATION IN THE ADULT NEWT,
NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS (RAFINESQUE)

by

Mary Therese Micaletti

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VITA

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INTRODUCTION

The newt and salamander have been used as a tool of study by many investigators. Under normal conditions, newt limbs regenerate when torn off or amputated. In the process of regeneration, a blastema, which is a mound of proliferating, undifferentiated fibroblasts, forms and gradually grows into a functional limb. The effects of various compounds on this regeneration blastema have been tested, including beryllium (Scheuing and Singer, 1957), cortisone (Manner, 1953), and Actinomycin-D (Carlson, 1965). Butler (1935) studied the effect of x-irradiation on the growth of the larval blastema.

Much controversy exists concerning the nature and pharmacological effects of the cyanide-containing glucoside, amygdalin. Amygdalin (Laetrile) has been used as a treatment and/or cure for cancer in Mexico, Canada, the Philippines, and Europe (Brown, 1974). Cancer cells are rich in β -glucosidase and β -glucuronidase, enzymes which split nitrilosides and liberate the toxic hydrogen cyanide (Krebs, 1970; Haisman and Knight, 1966). Compared to the liver, spleen, and kidney, cancer cells have low concentrations of rhodanese, an enzyme which detoxifies the hydrogen cyanide into thiocyanate (Rosenthal, 1948; Westley, 1973; Oke, 1969).

Blastema cells have been found to be rich in β -glucuronidase (Schmidt, 1966; 1968), and the occurrence of β -glucuronidase is paralleled by equal concentrations of β -glucosidase (Culbert, 1974). Also, blastema cells should be deficient in rhodanese, since rhodanese is proven to be deficient in proliferating, undifferentiated cells and cancer cells (Unsworth, 1975; Rosenthal, 1948).

If the blastema is composed of undifferentiated, embryonic-like cells, and if these cells are rich in β -glucosidase and deficient in rhodanese, then limb regeneration should be inhibited; since cells of the wound epidermis are not embryonic-like and not as actively proliferating, their growth should not be inhibited. Also, if amygdalin does inhibit cell proliferation of the blastema, this study could serve as the basis for other experiments, such as the effect of amygdalin on various proliferating cell types (e.g., tumors).

REVIEW OF RELATED LITERATURE

REGENERATION (GENERAL)

Lower vertebrates have an ability to regenerate that is far greater than that of higher vertebrates. Regeneration might be indicative of phylogenetic primitiveness, yet there are many phylogenetically low members of the animal kingdom that regenerate poorly if at all (Morgan, 1901). Schotte (1939) postulates that regeneration is a recapitulation of the developmental processes progressively restricted by age or increasing complexity and specialization. The most attractive view of regeneration is analogous to the developmental processes, for the differentiating tissues of the regenerating amphibian appear to rely on many molecular activities also found in developing organ anlage (Schmidt, 1968).

Spallanzini was a pioneer experimental morphologist who first formally described the regeneration phenomena in amphibians (Schmidt, 1968; Goss, 1969). Regeneration is the process by which organisms reconstitute lost parts of the body (Schmidt, 1966c). In amphibian regeneration, the original lost appendage is structurally and functionally duplicated. The basis of regeneration is the blastema, an anlage of unspecialized-appearing cells, which develops to

duplicate the original lost structure. The origin of the blastema resides within the amputation stump (Butler, 1935; Butler and Obrien, 1942; Chalkley, 1954). No regeneration occurs without a blastema. Although the origins are not actually embryonic, developmental processes are found in both repair and regeneration.

Regeneration occurs distal to the point of amputation. Stump tissues, such as skeleton, nerves, skin, and muscle, are continuous with the regenerating tissues. The regenerating limb becomes vascularized after appearance of a limb bud. The rate of regeneration depends on temperature, size of the appendage, age of the animal, season of the year, and the presence or absence of bone or cartilage (Schmidt, 1968).

Three main phases exist in the regeneration process. The first is the preblastemic phase, which is a slow postamputational phase. The second is the faster blastemic phase, a growth period during which the conic blastema forms. The third is the differentiative and morphogenetic phase, a slow maturation process (Schmidt, 1968).

Preblastemic

The trauma of amputating the forelimb of the newt triggers off responses by the tissues during the first 24 hours, for subapical tissues are shredded and broken by the shearing action of the scalpel. Within the first 2 to 3

minutes after amputation, blood clots at the wound surface. The substance of the clot may also serve as a substrate, for guidance in cellular closure of the wound (Schmidt, 1968). In less than 24 hours, cell migration from the perimeter of the injured epidermis closes the wound, forming an epithelium over the injured amputation surface. This process of wound closure is common in vertebrates. It occurs in mammals (Arey, 1932) as well as in amphibians (Lash, 1955). When the limb of the adult newt is amputated, the epidermis at the edge of the wound dissociates and cells release their desmosomal contacts with each other. Activated and mobile, these cells form a thin layer that glides over the wound between the blood clot and the injured tissue beneath. Although the cell migration appears ameboid, it is probably a sliding motion at the leading edge (Schmidt, 1968; Lash, 1955; 1956; Chihakulus, 1952). The wound is closed when the migrating cells meet centrally.

In this process of healing, an inflammation response persists for several days. Neutrophils and macrophages invade the clot to ingest foreign particles, fibrous and cellular debris of ruptured cells and tissues (Manner, 1953; Schmidt, 1966a). Granulocytes, erythrocytes and some lymphocytes from injured blood vessels are also prominent. These initial changes are similar to the inflammatory responses in injury of mammals. The eosinophilic granulocyte is particularly abundant, and may possibly increase

the viscosity of the edematous space (Schmidt, 1962a).

Distally, the amputation stump undergoes constant upheaval within the subepithelial tissue. Muscle is quickly depleted of its glycogen content (Schmidt, 1960). Edema may occur between the wound epithelium and the subjacent tissues. At first, the apical wound epithelium is translucent and shows blood underneath, either in vascular stasis or displaying very sluggish blood flow. Vascular flow soon increases and resumes a fairly normal rate. The apical epithelium appears blanched or pale yellow. Pigment cells may be present at this time, but are more typical of later stages (Schmidt, 1968).

Wound epithelium is thicker than the peripheral epidermis. The cells of the basal layer often lie perpendicular to those of the outer flattened layer (Schmidt, 1958b). Salpeter and Singer (1960) noticed cell-to-cell contacts between basal epithelial cells and subjacent cells. It is generally accepted that the basement membrane prominent under mature epidermis is absent under early wound epithelium (Schmidt, 1968). An infrequency of mitosis in cells of the wound epithelium greatly contrasts with the cell proliferation in the peripheral epidermis. This proliferation contributes cells to the wound epithelium.

Wound Epithelium

During the preblastemic phase, wound epithelium increases in thickness. Cells accumulate both by migrating from the peripheral epidermis and by mitotic activity within the wound epithelium. Basal cells conform to the irregularities of the substratum. About 10 days after amputation, the mitotic activity of the wound epithelium increases greatly (Chalkley, 1954), reaching a maximum of mitotic activity on the sixteenth day (Manner, 1953) to prepare more cells for a growing blastema.

The wound epithelium is described as serving a passive function (Nicholas, 1955); as a dumping ground for detritus (Singer and Salpeter, 1961); as a source of blastema cells (Hay, 1952; Rose and Rose, 1965); and as actively influencing the direction of the blastema (Thornton, 1965).

Direct contact between apical wound epithelium and underlying tissues is essential to regeneration (Schaxel, 1921; Schmidt, 1962a). Thornton and others (1954; 1956a; 1956b; 1957; 1960; 1965) have exemplified that apical wound epithelium directly controls the morphogenesis of the regenerating limb. If the wound epithelium is removed or selectively destroyed, no morphogenesis occurs (Thornton, 1957; 1958). Yet, if denuded limb stumps are covered with skin from other parts of the body, or inserted into a body

cavity (Deck, 1955; Goss, 1956), some histogenesis is observed. Thus, wound epithelium usually permits morphogenesis of the stump tissues, but is not essential for differentiation of individual tissues.

Striated Muscle

When the limb of the adult newt is amputated, the injured muscle fibers become frayed. Much of their sarcoplasmic glycogen is lost within the next few days, although the structural integrity of the muscle appears intact (Schmidt, 1958b; 1960; 1966c). During the first 48 hours, there is an increase in mitochondria within the injured muscle (Schmidt, 1966a). Sarcolysis, lipophanaerosis, and fibrillar disorientation and dislocation of muscle nuclei occur (Hay, 1959; Schmidt, 1958b). As a result, fibers become thin and separate from each other.

Hay (1962) noticed loss of the glycocalyx (the polysaccharide-protein basement membrane) of injured myofibers in larval urodeles, while Norman and Schmidt (1967) found the glycocalyx present, though convoluted, during regression of the myofibers of the adult newt.

During the next few weeks, considerable regression of the myofibers along with sarcolysis, lipophanaerosis, and macrophages occur even in the presence of the regeneration blastema (Schmidt, 1960; 1966a). It is concluded by Schmidt (1968) and Hay (1961) that differentiation of muscle into fibroblasts occurs.

Nerve

Nerves are clearly very important in amphibian regeneration, yet their role remains a mystery. Butler and Schotte (1941; 1959) discovered that transection of brachial nerves at any time before the regeneration blastema forms inhibits all regeneration. Denervation from the time of amputation up to the time of blastema formation usually resulted in regression or destruction of the regenerating Ambystoma limbs. The limbs become independent of this neural influence only when the blastema was about to enter the morphogenetic phase.

When brachial nerves are transected, they regress and then reconstitute. The amputated nerves distally undergo Wallerian degeneration. Like mammals, degeneration seldom extends proximally beyond the first two nodes of Ranvier (Schmidt, 1966a). From the second day of the pre-blastemic phase until the early differentiative phase, Schmidt (1966a) found much dissociation of nerve myelin and the presence of lipid-engorged macrophages. Although Singer (1949) reported axonal regeneration within the first few days after amputation, the earliest myelinization did not appear until the late preblastemic phase, while myelin degeneration was still noticeable.

Neural sheath cells (fibroblasts and Schwann cells) undergo mitosis, and daughter cells are free to move after injury. Distal migration of such "activated" cells was postulated as contributing to the regeneration blastema (Chalkley, 1954; Guyenat and Schotte, 1926).

The postulated trophic action of nerves in regeneration has been chemically investigated (e.g. with acetylcholine and sympathin) without notable success. Yet, evidence for the presence of a trophic agent which influences regeneration exists (Goss, 1969).

Skeleton

Osteocytes and chondrocytes which are released from their lacunae and modulate to their former fibroblastic state constitute an important source of the regeneration blastema (Hay, 1962; Schmidt, 1958b; Eggert, 1966; et al.).

For about 10 days after amputation, the bone terminus appears lifeless with empty enlarged lacunae and a few modulated osteocytes distally at the cut end (Schmidt, 1968). Osteoclasts of Howship's lacunae are noticeable. They are scavengers of bone, ingesting bone matrix (Gonzales and Karnovsky, 1961; Hancox, 1949; 1956) and embodying osteocytes freed from their lacunae (Bloom, Bloom, and McLean, 1941; Schmidt, 1958b; 1963). The fate

of the osteoclasts is unknown. It is only known that they function in the remodeling of bone and cartilage.

Bone erosion continues for 20 to 30 days after amputation. Then a cartilaginous collar forms around the injured skeleton. This cartilage develops distally in the overlying regeneration blastema to form new skeleton (Schmidt, 1958b).

Connective Tissue

The dermis and subepidermal basement membrane do not appear between the wound epithelium and subjacent tissues. This permits intimate contact between the epithelium and differentiating cartilage (Schmidt, 1958b; 1962a; 1966b; Schmidt and Weidman, 1964). It appears that the absence of the dermis contributes to the regeneration process.

It is reported that collagenase, along with other proteases and macrophages (Usuku and Gross, 1965) can dispose of unwanted collagen fibers which could interfere with the regeneration process. An enzyme that digests collagen protein may also retard the development of the dermis and basement membrane during regeneration by efficiently removing the extracellular fibers as fast as they are produced.

The fibrous components of the dermis of the adult newt are similar to those of all connective tissues. Schmidt (1962b; 1962c) observed collagen, reticular, and elastic fibers. Ide-Rozas (1937) and Mettetal (1939) observed mast cells, macrophages, melanocytes, fibroblasts, and fibrocytes.

The fibroblast cell is believed to be the source of collagen forming the lamellar basement membrane (Porter, 1964). The fibroblast has finely distributed nuclear chromatin with one or more distinguishable nucleoli. The cytoplasm is basophilic and usually abundant due to many ribonucleoprotein granules (ribosomes) which are either free or attached to the cisternal endoplasmic reticulum. The nucleus is large and rounded (Manner, 1953). The electron microscope shows mitochondria to be small and crowded around the nucleus, and the Golgi apparatus and centriole to be typical (Schmidt, 1968).

In studies of cultured fibroblasts, Porter and Pappas (1959) have reported the intracellular synthesis of tropocollagen, the protein unit of collagen and the extracellular condensation of these protein units into striated fibrils. Norman and Schmidt (1966; 1967), found intracellular fibrils in the regeneration limb of the newt. This may imply that fibroblasts synthesize and condense collagen protein into fibrils completely within the cell. The fibroblast also synthesizes mucopolysacchar-

ides, e.g. hyaluronic acid, to be secreted into the extracellular space (Kennedy, 1960; Crane, 1962). This contributes to the ground substance (Schmidt, 1968).

According to most investigators (Grillo, 1964; DeVito, 1965; Douglas, 1963; Russle and Billingham, 1962; et al.) the fibroblast is the principal cell forming granulation tissue of repairing skin wounds in mammals and other vertebrates. In amphibian regeneration, which is a much more extensive process than cutaneous repair, the granulation tissue is known as the blastema and is thought to possess embryonic properties (Hay, 1959; Schotte, 1939).

From as early as Barfurth (1891) and more recently by Manner (1953) and Schmidt (1966c) it was concluded that fibroblasts of connective tissue proliferate and migrate to form the regeneration blastema of amphibians. Influences supporting a connective tissue origin of the blastema can also be made from investigations of Chalkley (1954; 1959).

Blastemic Phase

A bulb-shaped regeneration blastema is recognizable about 10 to 15 days after amputation of the newt limb. Within the next 10 days, the blastema grows to several times its original size, and changes its morphology; the apex narrows in circumference, and the whole structure takes on a conical appearance. This growth into a sizable

conic protuberance is very critical, because it is this advanced blastema that serves as the foundation for further morphogenesis into a new limb.

Origin of the Blastema

Blastema cells of leucocyte origin were reported by Hellmich (1930) and Ide-Rozas (1937). Macrophages (Ide-Rozas, 1937; Trampusch and Harrebomee, 1965), mesenchymatous embryonic cells, wandering cells and reserve cells (Hellmich, 1930; Ide-Rozas, 1937; Weiss, 1939) were also reported as transforming into blastema cells. Rose (1964) and Hay (1952) postulated the epidermis as the major contributor to the regeneration blastema.

Striated muscle (Hay and Fishman, 1961; Holtzer, 1961; Thornton, 1962; Trampusch and Harrebomee, 1965), by dedifferentiating into embryonic mesenchymal cells (Hay, 1959), was named a source of blastema cells. Also, dedifferentiating skeletal tissue was strongly recommended as a source of blastemal cells (Butler, 1933; Foret, 1966; Hay, 1962; Trampusch and Harrebomee, 1965), for cartilaginous and bony tissue are very close to their fibroblastic origins.

A hematogenic origin of the blastema has been disproven. Regeneration was totally inhibited when the adult limb was x-irradiated before amputation (Butler, 1931; 1933; 1935). Although Butler protected the major hemopoietic tissues from irradiation, the circulating leukocytes

did not promote regeneration.

There is also evidence against an epidermal origin of the blastema. In 1954, Chalkley showed that cell division begins in the inner limb tissues much earlier and more proximally than ever realized. This could account for the appearance of a blastema of non-epidermal origin.

Schmidt (1962a; 1966c; 1968) strongly advocates the connective tissue of the amputation stump (which includes sheaths of the skeleton, muscle fibers, nerves, and blood vessels, and also the chondrocytes and osteocytes freed from their lacunae during injury) as the sole source of blastemal cells. Microscopic examinations show "streams of cells" of the regenerating limb migrating toward the apical wound epithelium in blastema formation. The blastema cell has been described as mesenchymal and embryonic. Salter and Singer (1960) found 80% of the blastema cells to possess an extensive rough-surfaced endoplasmic reticulum, a large nucleus with a distinct double membrane, a nuclear envelope with pores, and a prominent nucleolus. Norman and Schmidt (1967) found protoplasmic extensions with many free ribosomes and a well-formed Golgi complex. Most logically, the "activated fibroblasts" are the cells of the connective tissue that proliferate and migrate to form the regeneration blastema (Schmidt, 1968).

Since a fibroblastic origin of the blastema is proposed, the potentialities of the blastema cells should

be examined.

If the blastema is an embryonic cell as postulated by Schotte (1939) and Hay (1958; 1959), then this cell must be able to differentiate into several tissues of mesenchymal origin (e.g., muscle, connective tissue, cartilage, bone, and blood vessels). Most investigators now accept the theory that the blastema arises by a transformation of cells composing muscle, cartilage, and other inner tissues of the limb stump into mesenchyme-like cells (Thornton, 1938; Schotte, 1939).

Hay and Fishman (1961) used tritiated thymidine, a nucleoside incorporated into DNA, to determine cell proliferation and migration in blastema formation during limb regeneration in Triturus viridescens viridescens (Notophthalmus viridescens viridescens). In the first of a 3-part series of experiments, the location and number of cells engaged in DNA synthesis during blastema formation was determined by fixing regenerates on the day they received tritiated thymidine. In the next 2 series, the regenerating limbs were followed at daily intervals after treatment with radioactive thymidine. Muscle and connective tissue were traced in the first group, while blood and epithelium were traced in the second group.

The results of this experiment confirmed Chalkley's postulation (1954), that dedifferentiation in blastema formation is accompanied by cell proliferation. Cells of

the muscle, endomysium, periosteum, nerve sheaths, and loose connective tissue of the distal limb stump begin to synthesize DNA 4 to 5 days after amputation at a distance of 1 mm proximal to the amputation surface. The number of cells synthesizing DNA in the inner tissues increases rapidly 10 to 20 days after amputation. Epithelium of the amputation surface attains a high level of DNA synthesis 8 days after amputation, and its cells migrate distally to increase the size of the apical cap 10 to 15 days after amputation. During blastema formation, only 2% of the cells in the apical cap incorporate thymidine, and these do so only slightly. After blastema formation, the regenerating limb starts to elongate and the apical cap thins out as its cells resume synthesizing DNA. In the inner stump tissues, the presence of DNA synthesis shows that dedifferentiating cells are not degenerating. Living cells are released from the confines of their previous environment and become mitotically active. Chalkley (1954) and Hay and Fishman (1961) showed that cell division in the proximal internal tissues begins early enough to account for the presence of a blastema without postulating a purely epidermal, hematogenic, or fibroblastic origin.

Differentiative and Morphogenetic Phase

On approximately the twenty-sixth day, the conic

blastema (now with a pointed apex and a broad base on the limb stump) starts to flatten dorso-ventrally and evolves into a paddleform regenerate. This is also called a conic paddle regenerate by Schmidt (1958a). This stage lasts only two days, after which time a groove forms to outline the first two digits. This digitform regenerate acquires all its digits within a few days.

The final degree of morphogenesis of the regenerate approaches, but often does not equal that of the lost original limb. Nevertheless, the regenerate is completely as functional as the original limb.

AMYGDALIN

Chemical Characteristics

Amygdalin is the common generic name for D-mandelonitrile- β -gentiobioside (gentiobioside is a disaccharide with a β (1,6) glycosidic linkage) or D-mandelonitrile- β -D glucosido-6- β -D-glucoside (Merck Index, 1976). Because this glycogen compound structurally contains the $C\equiv N$ (nitrile) bond, it is described as a type of nitrilose, a term proposed by Ernst T. Krebs, Jr., to include all cyanophoriglycosides of dietary significance. Nitriloses are a group of essentially non-toxic, sugary compounds found in over 800 plant types, many of which are edible (Krebs, 1970).

Amygdalin is one of the most common nitrilosides. It is white, crystalline, water-soluble (1 gram of trihydrate dissolves in 12 mls water), slightly bittersweet, and has the taste and odor of bitter almond (Oke, 1969; Burk, 1975). The pH of a saturated water solution is about 7. The molecular weight of amygdalin is 457.42 grams; 5.69% of this weight is cyanide (Merck Index, 1976).

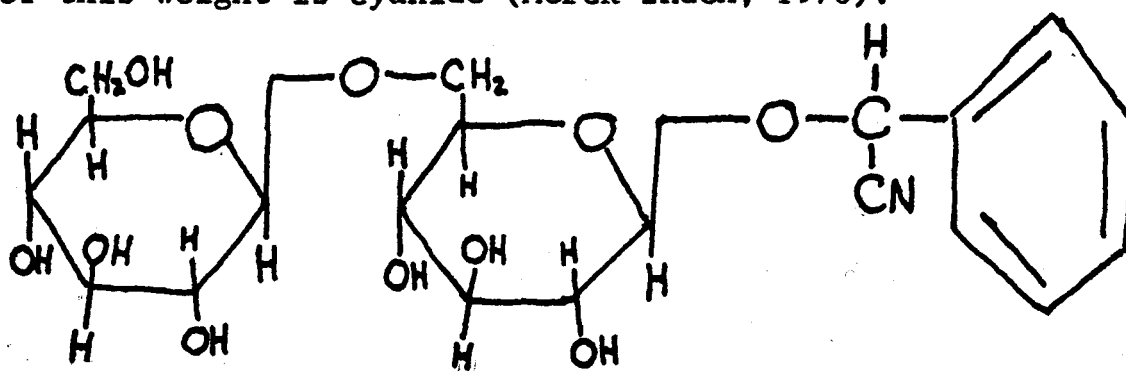


Figure 1: Structure of Amygdalin ($C_{20}H_{27}NO_{11}$)

Occurrence in Nature

Cyanide in trace amounts is almost ubiquitous in the plant kingdom, occurring mainly in the form of cyanogenetic glycosides. Almost all the sorghums, some grasses (e.g. Johnson and Sudan), the clovers, minna grass, the flax plants, and the desert almond produce hydrocyanic acid. Cyanogenetic glycosides are also found in foods commonly consumed by man, such as maize, sorghum,

millet, field bean, lima and kidney beans, the sweet potato, cassava, lettuce, and linseed almond. It is also in the seeds of lemons, limes, cherries, apples, apricots, prunes, pears, and plums (Oke, 1969; Krebs, 1970).

Table 1 (Montgomery, 1969)
Cyanide Yield in Some Plants

Plant	HCN yield, mg/100 g	Reference
Bitter almond	250	Polson & Tattersal (1959)
Sorghum	250	Anonymous (1903)
Bitter cassava		
---dried root cortex	245	Collens (1915)
---stem	113	Collens (1915)
---whole root	55	Clark (1936)
Lima bean		
---Java, colored	312	Guignard (1907)
---Arizona, colored	17	Montgomery (1964)
---American, white	10	Montgomery (1964)
---Burma, white	210	Kohn-Abrest (1906)
Linseed	53	Auld (1913)
Kidney or navy bean	2	Montgomery (1969)
Garden pea	2	Montgomery (1969)

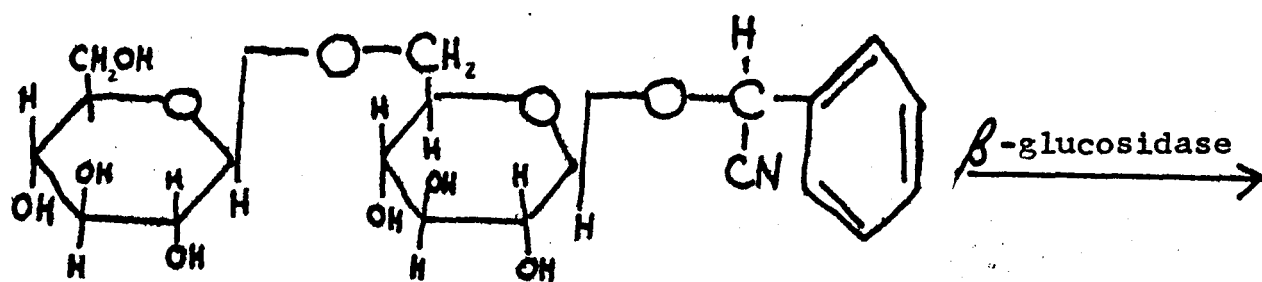
One of the richest sources is the defatted apricot pit, which contains 4000 mg HCN/100 g (Krebs, 1970).

Since primitive times, these cyanide-containing plants have been eaten by man and were a constituent of the diet of wild herbivores, carnivores, and domesticated sheep and cattle.

Non-toxicity

Despite the fact that cyanide is a component of amygdalin, amygdalin was found to be very non-toxic to animals. From 500-2500 mg amygdalin/ kg body weight were injected intramuscularly into mice with no evidence of toxicity (Manner, DiSanti, and Michaelson, 1976). Burk, McNaughton, and von Ardenne (1969) reported 5-10 grams/kg given by various routes. The Sloan-Kettering Cancer Institute found improvement of cancerous mice upon treatment with 1-2 grams crystalline amygdalin/ kg body weight/ day (Burk, 1975). Doses of 500 mg were given intravenously on alternate days for a total of 6 injections (Browne, 1974), and dosages of 250 mg/kg (i.p.) (Lea et al., 1975) were also reported therapeutic and non-toxic.

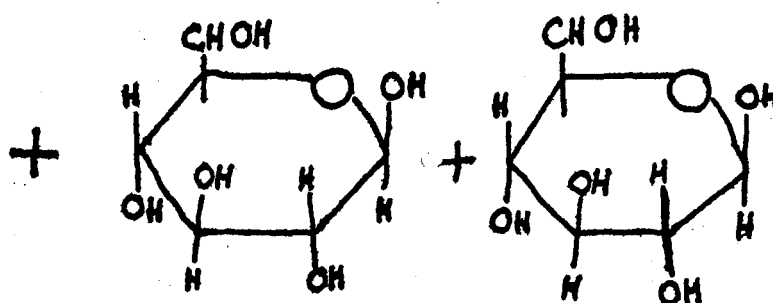
The non-toxicity of amygdalin is due to the manner in which it is broken down.



Amygdalin



Mandelonitrile



2 Glucose

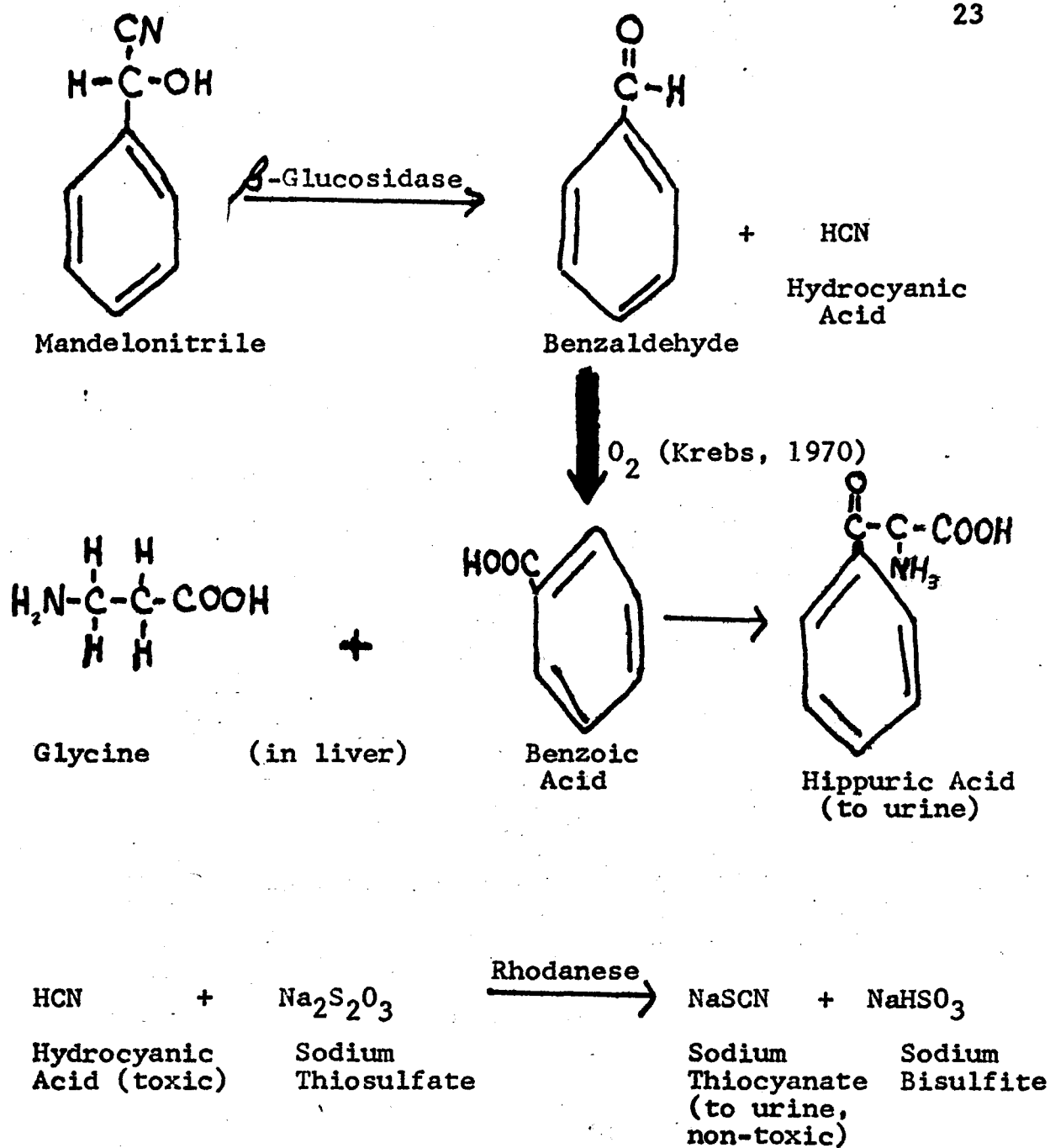


Figure 2:

(Rosenthal, 1948)
 (Sorbo, 1953)
 (Krebs, 1970)
 (Oke, 1969)
 (Montgomery, 1969)

β -GLUCURONIDASE

β -glucuronidase (β -D-glucuronide glucuronohydrolase, EC 3.2.1.31) is a hydrolytic enzyme located in the cell lysosome bodies. It is widespread in animal tissue. It plays a role in the catabolism of mucopolysaccharides (the carbohydrate-protein that forms ground substance) (Levy and Marsh, 1960), and is also associated with an increase in cell proliferation (Kerr, Campbell, and Levy, 1949) and tumor metabolism (Fishman, Baker, and Borges, 1959).

Stolk (1962) examined skin wounds of the axoltl for the presence of β -glucuronidase. Five days after wounding, there was no activity in the superficial dermis, although the adjacent leucocytic inflammation was very active for the enzyme. With the onset of healing, proliferating fibroblasts were moderately active for β -glucuronidase.

Dukiet and Niwelinski (1960) investigated β -glucuronidase activity of the regenerating limb of Triturus alpestris (Notophthalmus alpestris). The limb musculature was enzyme reactive except at the injured terminals. The periosteum was intensely reactive. Forming blastema cells were active for β -glucuronidase as early as 5 days after amputation, and at 10 days the activity of the growing blastema was noticeably increased. Chondrogenesis began at the end of the second week, and the chondroblasts were grad -

usually reactive for β -glucuronidase. Regenerating striated muscle was more enzyme active than cartilage. Blastema cells at the tip of the cartilaginous digital skeleton show intense reactivity.

RHODANESE

Rhodanese (thiosulfate: cyanide sulfurtransferase, EC 2.8.1.1) is a mitochondrial enzyme which catalyzes the formation of thiocyanate from thiosulfate and cyanide (Lang, 1932; Sorbo, 1953; Westley, 1973). This enzyme thus possesses a cyanide-detoxifying function.

Rhodanese is widespread in the animal kingdom. It is found in all mammalian tissues except blood and muscle. High concentrations are found especially in liver, adrenal glands, and kidney, with lower concentrations in the brain. Less than 1% of rhodanese is associated with connective tissue (Westley, 1973).

Smaller concentrations of rhodanese have been found in tumorous and proliferating tissue than in normal tissue. Rosenthal (1955) found hepatomas to contain only 20% of the rhodanese concentration of normal liver. Mendel, Rudney, and Browman (1946) reported that rhodanese was absent in transplanted mouse sarcomas and normal mouse jejunal mucosa (Rosenthal, 1948). Unsworth (1975), in experimenting with the embryonic development of mouse liver and kidney, found that rhodanese levels correlated (increased)

with the onset of organogenesis.

Rhodanese activity has been found to be higher in amphibians than in humans, dogs, or fowl. Since rhodanese is also present in many bacteria and plants, Schievelbein et al. (1969) felt that rhodanese arose early in evolutionary history, when HCN was prevalent.

MATERIALS AND METHODS

One hundred aquatic newts, Notophthalmus viridescens viridescens (Rafinesque), were used in this study. Their average weight was 1.8 ± 0.06 grams.

The newts were acclimated to the laboratory for one month. For the first two weeks all were placed in a 10-gallon fish tank filled with aerated dechlorinated tap water at a temperature of $23 \pm 2^{\circ}\text{C}$ ($72.2 \pm 2^{\circ}\text{F}$). From this time onwards, the newts were placed in fingerbowls of $4\frac{1}{2}$ inch diameter. The fingerbowls were filled with 125 cc dechlorinated tap water at $23 \pm 2^{\circ}\text{C}$. Room temperature was maintained at $23 \pm 2^{\circ}\text{C}$. The water was changed twice weekly, and the newts were force fed Tubifex (redworms) or beef liver twice weekly (they were force fed by waving the food on a forceps in front of the newts face, for newts will not pursue food unless it is moving).

Newts 1-50 were designated as the experimental group, and newts 51-100 were the control group. Each newt was placed in a fingerbowl labeled "control" or "experimental", two newts per fingerbowl. The bowls were covered with a netting to prevent escape or transfer to another bowl.

On the zero day of experimentation, the right forelimb of all 100 newts was amputated with a sharp scalpel through the proximal third of the radius and ulna. Immed-

ately after amputation, and continuing daily for the next 14 days, injections were administered to the newts. The control group (51-100) was injected intraperitoneally with 0.05 cc isotonic amphibian ringers solution, a physiological saline solution which has a pH of 7.8 and is satisfactory to adult amphibian tissue. (See Table II)

Table II (Rugh, 1965)

AMPHIBIAN RINGER'S SOLUTION

NaCl.....	0.66 grams
KCL.....	0.015 "
CaCl.....	0.015 "
NaHCO (Buffer).....	0.030 " *
Distilled water.....	100 cc

* This is the amount necessary to regulate the pH at 7.8

The experimental group (1-50) was injected intraperitoneally with 0.05 cc amygdalin solution, which consisted of amygdalin dissolved in isotonic amphibian ringers solution. The pH of a saturated water solution of amygdalin is about 7; amygdalin dissolved in isotonic amphibian ringers solution however shows a pH of approximately 7.8. The amygdalin solution was at a dosage of 2000 mg/kg body weight (This dosage was found to be effective and non-toxic at the

Sloan-Kettering Institute (Burk, 1975)) to a 1.8 gram newt as shown by the following:

$$\frac{X \text{ mg amygdalin}}{0.0018 \text{ kg body wt.}} = \frac{2000 \text{ mg amygdalin}}{1 \text{ kg body weight}}$$

$$X = 3.6 \text{ mg/ injection needed.}$$

$$(0.05 \text{ cc}) (X \text{ mg}) = 3.6 \text{ mg/injection}$$

$$X = 72 \text{ mg/cc}$$

$$X = 0.072 \text{ g/cc}$$

$$\frac{0.072 \text{ grams}}{1 \text{ cc}} = \frac{1 \text{ gram}}{X \text{ cc}}$$

$$X = 13.89 \text{ cc}$$

Thus, 1 gram of amygdalin dissolved in 13.89 cc amphibian ringers solution will give a dosage of 2000 mg/kg. In this experiment, the amygdalin solution was made fresh daily. Following the proportion of 1 gram per 13.89 cc, 0.2 grams amygdalin was dissolved in 2.78 ml amphibian ringers solution.

Injections were administered with a disposable 1 cc Plastipak tuberculin syringe and 27½ g sterile disposable Yale hypodermic needle. After every injection, the needle was sterilized with alcohol. A new needle was placed on the syringe after every 12 injections, and a different syringe was used for the experimental and control groups.

On the sixteenth day, the right limb of each newt was re-amputated at the mid-humerus. The limbs were immediately placed in Bouin's fixative (See Table III), which is necessary to decalcify the long bones of the limb and preserve the tissue. After fixation of at least three days, the limbs were dehydrated by being passed through a series of ascending alcohols (70%, 80%, 90%, 95% and absolute) for a period of one hour per alcohol. They were then placed in toluene for 25 minutes. This time is crucial: the time must be long enough to "clear" the tissue, i.e., make the tissue soluble in paraffin (tissue is cleared when it becomes translucent), yet not so long that the tissue becomes brittle.

After toluene, the limbs were directly transferred to a container of melted paraffin (Tissue Prep, m.p. $56.5 \pm 0.5^{\circ}\text{C}$) which was placed in a Precision Thelco vacuum oven. They were infiltrated at $61 \pm 3^{\circ}\text{C}$ and 15 p.s.i. for a total of three hours. The paraffin was changed each hour to rid of any residual toluene.

After infiltration with paraffin, the limbs were positioned in a Tissue-Tek block mold (previously sprayed with Tissue-Tek mold release), fitted with a Tissue-Tek embedding ring, and filled with melted paraffin. The paraffin block was hardened fully, trimmed to just fit the dimensions of the limb, and secured in a Spencer "820" rotary

Table III (Humason, 1962)

BOUIN'S FIXATIVE

Picric acid, saturated aqueous.....	75.0 ml
Formalin.....	25.0 ml
Glacial acetic acid.....	5.0 ml

microtome. The microtome was adjusted to cut tissue sections 10 micrometers thick. A microtome knife, previously sharpened for two hours, was clamped into position, and the lower edge of the paraffin block was exactly parallel to the knife edge. Each section will have its upper edge cut along the front edge of the cutting knife. When the next section is cut, its lower edge will fuse with the upper edge of the previous section. This is due to the friction of cutting which slightly melts the paraffin at that point; thus, a ribbon is formed. The limb was positioned in the mold so that as the block was sliced, serial sagittal sections would result. The reason for this was so that the entire limb and blastema would be shown in one sagittal section.

The ribbon was partitioned into groups of approximately eight tissue sections; two groups of eight were placed on a microscope slide previously coated with Mayers albumin (for tissue adhesion) and then distilled water (to "float" the tissue section). The slides were then put on a hot-plate to eliminate "wrinkling" of the tissue sections and to induce flattening and drying.

When completely dried (about one day), the slides were stained with Mallory's triple connective-tissue stain (See Table IV) by being passed through the following solutions in the order and time indicated:

Table IV (Guyer, 1947)

MALLORY'S TRIPLE CONNECTIVE-TISSUE STAINMallory I

Acid fuchsin.....0.2 gram

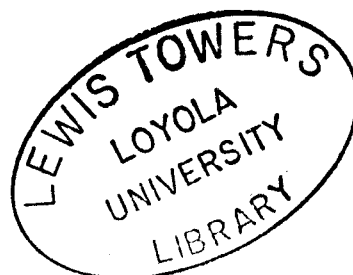
Distilled water.....100 cc

Mallory II

Aniline blue (grubler's water soluble).....0.5 gram

Orange G (grubler).....2.0 grams

Phosphomolybdic acid, 1% aqueous solution..100 cc



- 1). Xylene.....10 min.
- 2). Absolute alcohol..... 5 min.
- 3). 95% alcohol..... 3 min.
- 4). 80% alcohol..... 1 min.
- 5). 70% alcohol..... 1 min.
- 6). 50% alcohol..... 1 min.
- 7). 35% alcohol..... 1 min.
- 8). Distilled water..... 1 min.
- 9). Mallory I15 sec.
- 10). Mallory II 3 min.
- 11). 90% alcohol..... 1 min.
- 12). 95% alcohol..... 3 min.
- 13). Absolute alcohol..... 5 min.
- 14). Xylene.....10 min.

With this procedure, connective tissue, cartilage, and bone are stained blue. Nuclei, cytoplasm, fibroblasts, and muscle are colored red, and blood cells are yellow-orange.

When the slides were stained and dried, Pro-Texx mounting medium and a coverslip were applied to each. Tissue sections were then examined microscopically.

The following data was taken:

- (1) size of the blastema (in micrometers) was analyzed by measuring the distance from the tip of the radius and ulna to the base of the epidermis. For each limb,

four measurements were taken, one from the right and left side of the tip of each bone. Measurements were taken from the serial section in which the radius and ulna came closest to the epidermis (See Figure 3).

- (2) thickness of the epidermis at the level of the radius and ulna was measured in two ways. First, the thickest part of the epidermis was measured in micrometers. Secondly, the epidermis was measured as to its cell thickness (Figure 3).
- (3) descriptive histological observations were made
- (4) photographs were taken
- (5) all data was statistically analyzed.

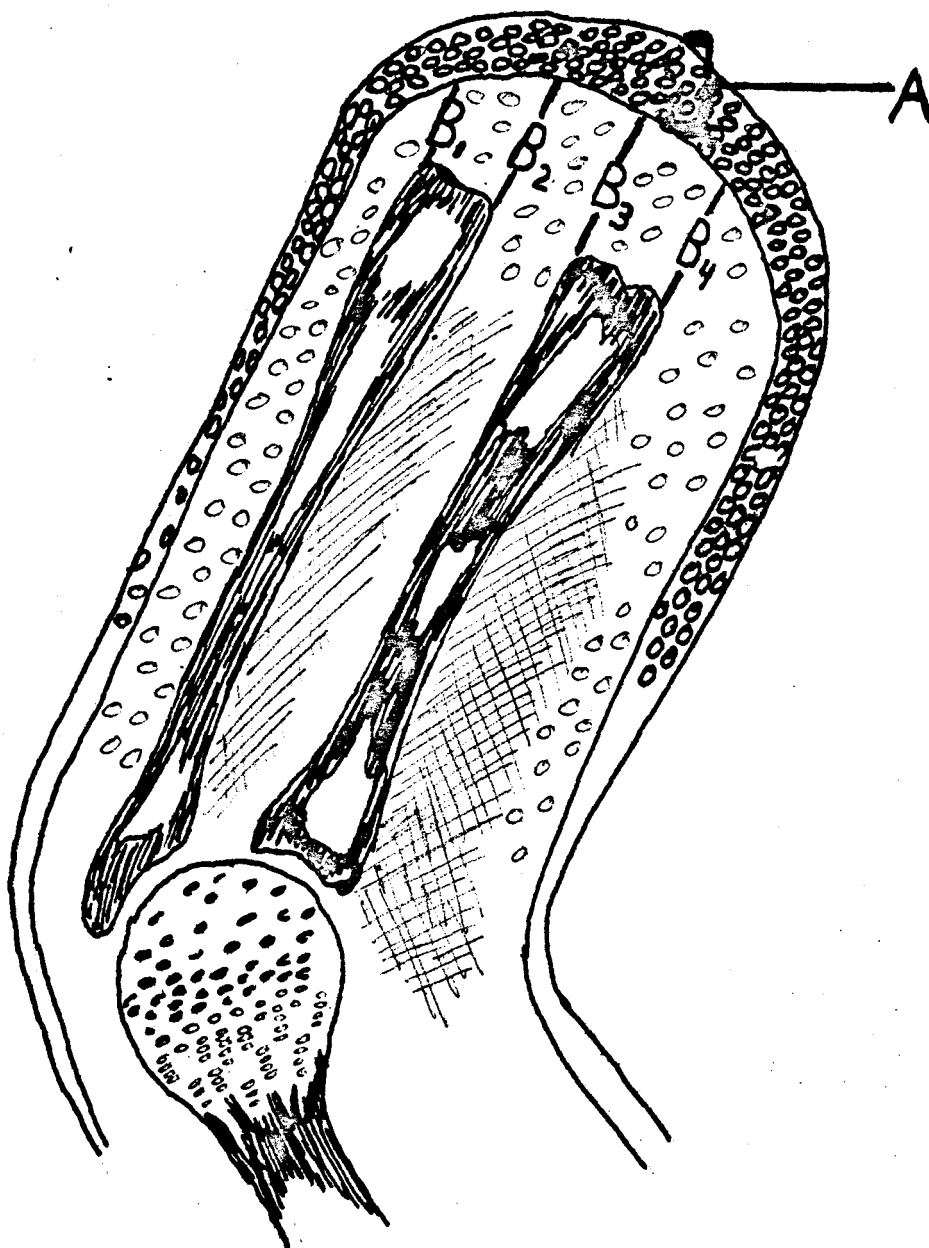


Figure 3:

- A.** Thickness of epidermis at thickest point
 1) in number of micrometers thick
 2) in number of cells thick

B₁, B₂, B₃, B₄. Size of the blastema measured

by finding the distance from the tip of the radius and ulna to the epidermis. Average calculated for each limb.

EXPERIMENTAL DATA

Amygdalin showed no effect on the thickness of the epidermis. Mean epidermal thickness of the experimental group was 82.00 ± 28.38 micrometers, which is an insignificant difference compared to the 85.59 ± 29.77 micrometers of the control group. If measured in cellular thickness, epidermal size was also similar; the control group had a mean of 7.32 ± 2.71 cells thick, while the experimental group's cell thickness was 6.84 ± 2.34 . This is an insignificant difference as calculated by the student t-test for independent means.

Amygdalin seemed to exert no effect on the stump tissues; as far as the 16th day, there was no observable difference in bone, muscle, or cartilage between the two groups.

The growth and cell constituency of the blastema, however, was significantly affected by amygdalin. The average blastema size of the experimental group was only 110.84 ± 57.89 micrometers, in comparison to the mean of 266.25 ± 87.69 micrometers for the control group. Many amygdalin-injected newts had no blastema at all, and the tip of the radius and ulna came right up to the base of the epidermis. In other amygdalin-injected newts, there

was an appreciable distance between the tip of the radius and ulna and the base of the epidermis. Histological observation showed this to be not a blastema, however, but rather a pseudoblastema composed of quiescent fibrocytes (instead of the normal proliferating fibroblasts). Edema was also prevalent in the experimental group.

In order to compare the control and experimental groups, the mean, standard deviation, and student t-test for independent means were calculated for each of the following categories: thickness of the epidermis in micrometers, cellular thickness of the epidermis, and size of the blastema in micrometers. A sample of each calculation type is given below.

Mean

The mean (\bar{X}) is the most common measure of the central tendency of a group of scores. $\bar{X} = \frac{\sum X}{N}$, where X signifies the scores in the distribution, N signifies the number of scores, and \sum instructs one to add all the scores.

E.g., in calculating the cellular thickness of the epidermis of the experimental group,

X = the number of cells thick of the epidermis
of each newt

N = the number of newts used

\bar{X} = the mean cellular thickness of the epidermis

$$\Sigma X = 301$$

$$N = 44$$

$$\bar{X} = \frac{301}{44} = 6.84$$

Standard Deviation

The standard deviation (S.D.) is the positive square root of the variance. Variance (s^2), which is an index of variability, is defined by the following formula:

$$s^2 = \frac{N\Sigma X^2 - (\Sigma X)^2}{N(N-1)}$$

In calculating cell thickness for the experimental group,

$$N = 44$$

$$\Sigma X^2 = 2295$$

$$(\Sigma X)^2 = (301)^2 = 90601$$

$$s^2 = \frac{44(2295) - 90601}{(44)(43)}$$

$$s^2 = \frac{100980 - 90601}{1892} = 5.48$$

$$S.D. = \sqrt{5.48}$$

$$S.D. = 2.34$$

Student t-test Between Independent Means

In this significance test, the null hypothesis (H_0), which states that the population mean for the experimental group is approximately equal to the population mean for the control group (that amygdalin has no effect), is tentatively held to be true. Data is collected and calculations are

done to see whether the results are likely to occur within the expected range of sampling error. If the observed mean differs by an amount which is not attributable to sampling error alone, then the drug can be postulated to have a significant effect. Thus, the alternative hypothesis (H_1), that the population mean of the experimental group is less than the population mean of the control group can be assumed. E.g., in order to see if the mean blastema size of the experimental group differed significantly from that of the control group, the following procedure is done.

Hypothesis

$$H_0: \mu_E = \mu_C$$

μ_C = population mean of the control group

$$H_1: \mu_E < \mu_C$$

μ_E = population mean of experimental group

Assumptions

1. The newts are independently and randomly sampled.
2. The groups are independent.
3. The population variances are homogenous.
4. The population distribution of $\bar{X}_C - \bar{X}_E$ is normal in form.

Decision Rules

Given: a directional test, a 0.05 level of significance and $N_1 + N_2 - 2 = 47 + 44 - 2 = 89$

degrees of freedom

If $-1.671 < t_{obs} < 1.671$, do not reject H_0

If $t_{obs} \leq -1.671$ or if $t_{obs} \geq 1.671$, reject H_0

ComputationControl Group (Group 1)

$$\bar{X}_1 = 266.25$$

$$s_1^2 = 7689.5$$

$$N_1 = 47$$

Experimental Group (Group 2)

$$\bar{X}_2 = 110.84$$

$$s_2^2 = 3351.2$$

$$N_2 = 44$$

$$t_{abs} = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{\left[\frac{(N_1 - 1)s_1^2 + (N_2 - 1)s_2^2}{N_1 + N_2 - 2} \right] \cdot \left[\frac{1}{N_1} + \frac{1}{N_2} \right]}}$$

$$t_{abs} = \frac{266.25 - 110.84}{\sqrt{\left[\frac{(47-1)(7689.5) + (44-1)(3351.2)}{44 + 47 - 2} \right] \left[\frac{1}{47} + \frac{1}{44} \right]}}$$

$$t_{abs} = 9.906$$

Decision

Reject H_0

The difference in mean blastema size was found to be significant even up to the 0.0005 level of significance. Computational equations and critical values of t are from Mc Call (1975).

Table V

Cellular Thickness of Epidermis

	No. of Newts	Mean + S.D.
Control	47	7.32 + 2.71
Experimental	44	6.84 + 2.34

At 0.05 significance level, a directional test, and 89 df,
 $t = 0.9017$. Critical value of $t = 1.671$.

$-1.671 < t_{\text{obs}} < 1.671$. Do not reject H_0 .

Thickness of Epidermis in Micrometers

	No. of Newts	Mean + S.D.
Control	47	85.59 + 29.77
Experimental	45	82.00 + 28.38

At 0.05 significance level, a directional test, and 90 df,
 $t = 0.5895$. Critical value of $t = 1.658$.

$-1.658 < t_{\text{obs}} < 1.658$. Do not reject H_0 .

Size of Blastema in Micrometers

	No. of Newts	Mean + S.D.
Control	47	266.25 + 87.69
Experimental	44	110.84 + 57.89

At 0.05 significance level, a directional test, and 89 df,
 $t = 9.906$. Critical value of $t = 1.671$. $t > 1.671$.

Reject H_0 .

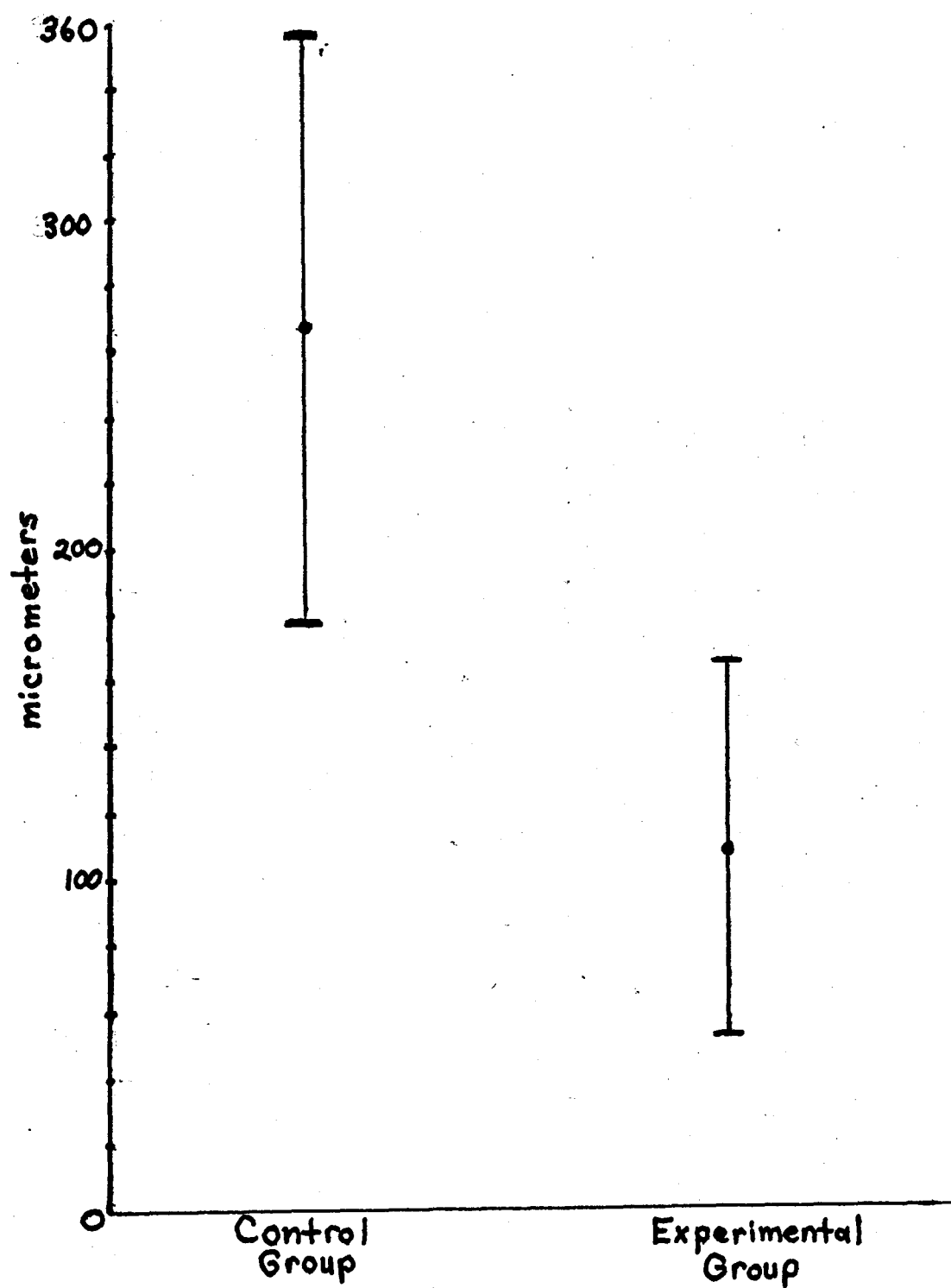


Figure 4 : Size of the Blastema

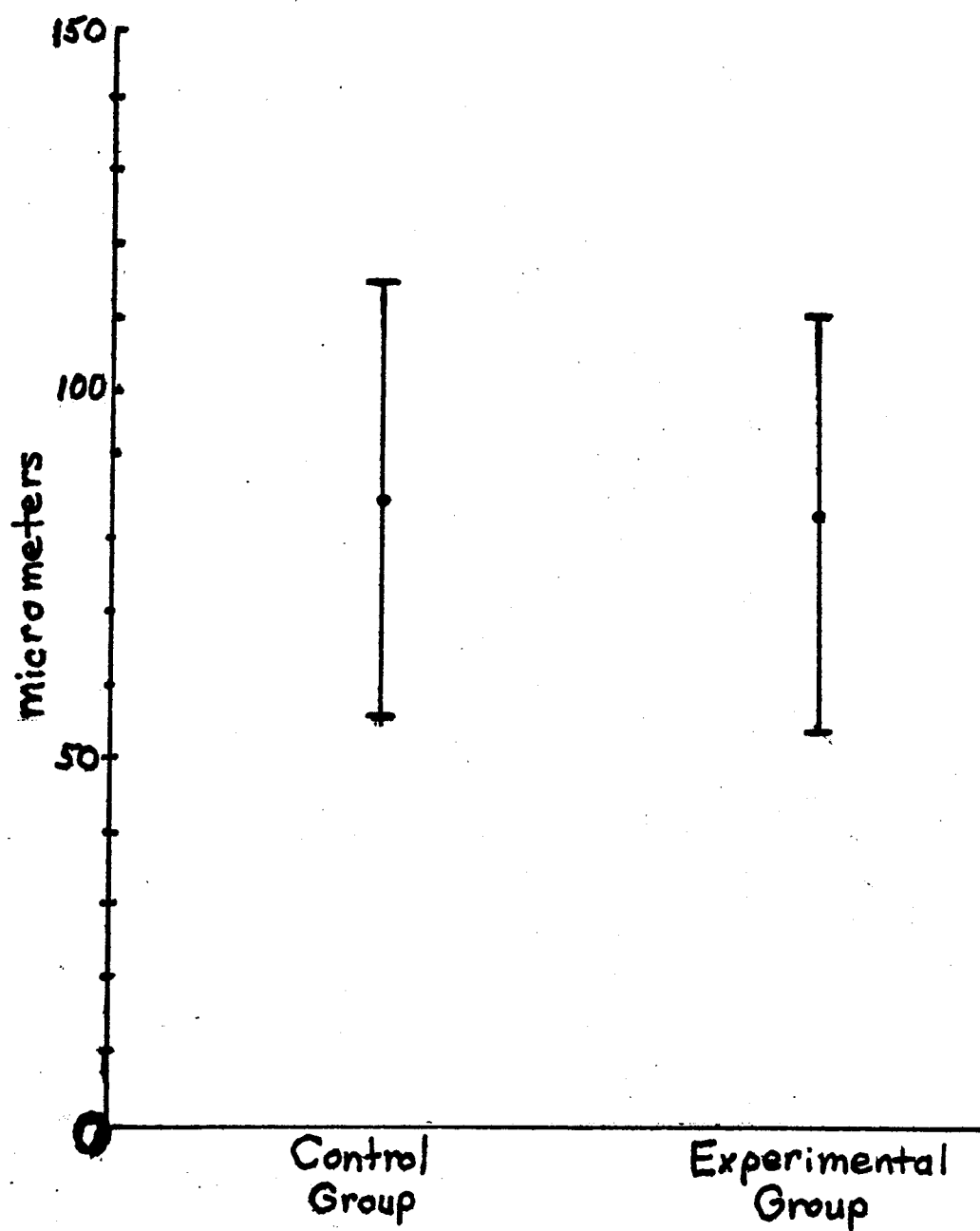


Figure 5 : Thickness of Epidermis

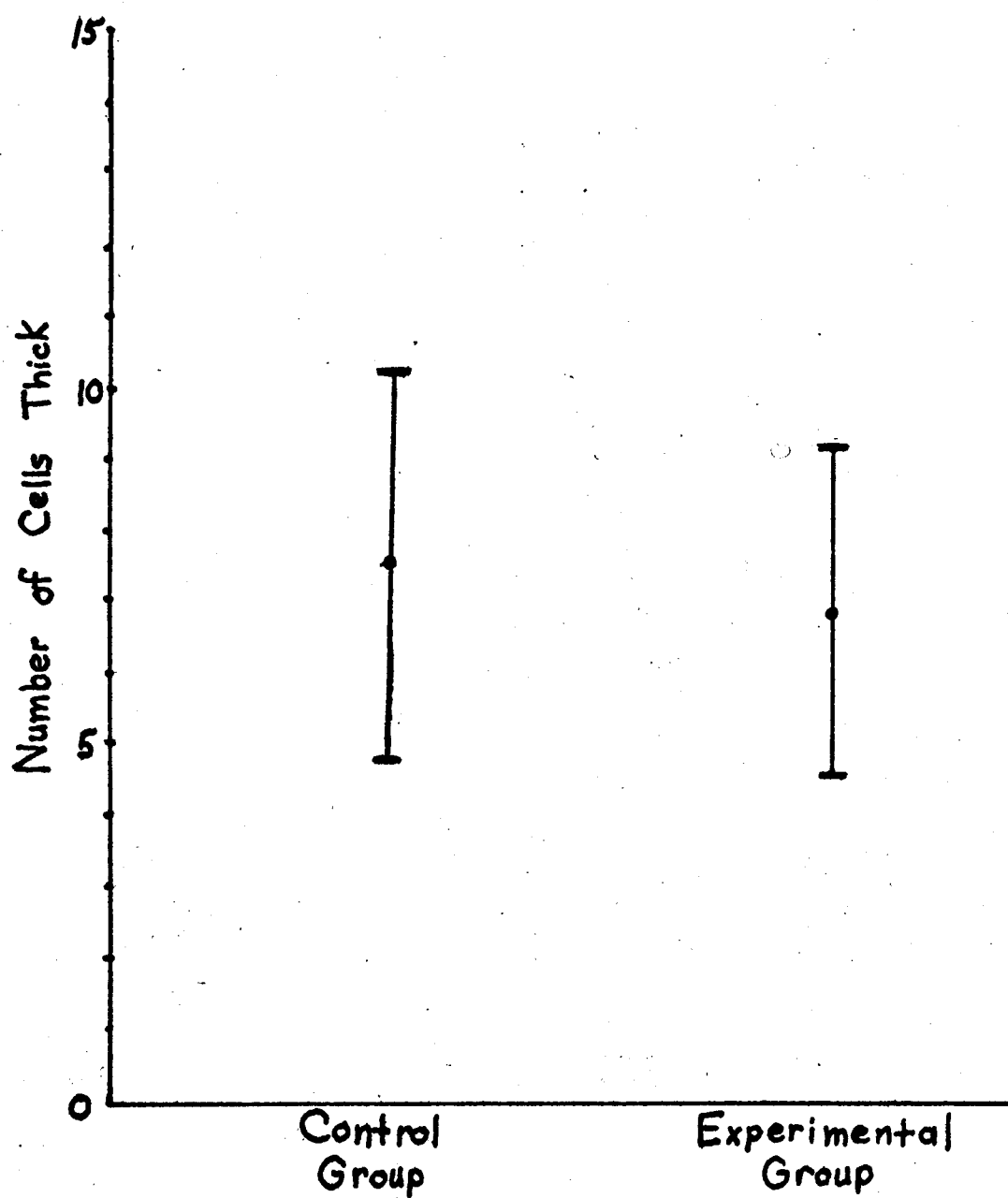


Figure 6 : Thickness of Epidermis

Figure 7: The control limb 16 days after amputation (25X).

**Figure 8: Limb of amygdalin-injected newt 16 days
after amputation (25X).**



Figure 7



Figure 8

Figure 9: Higher power of control limb. Note the regular, organized arrangement of fibroblasts (40X).

Figure 10: Experimental limb. Radius and ulna are covered only by wound epidermis (40X).

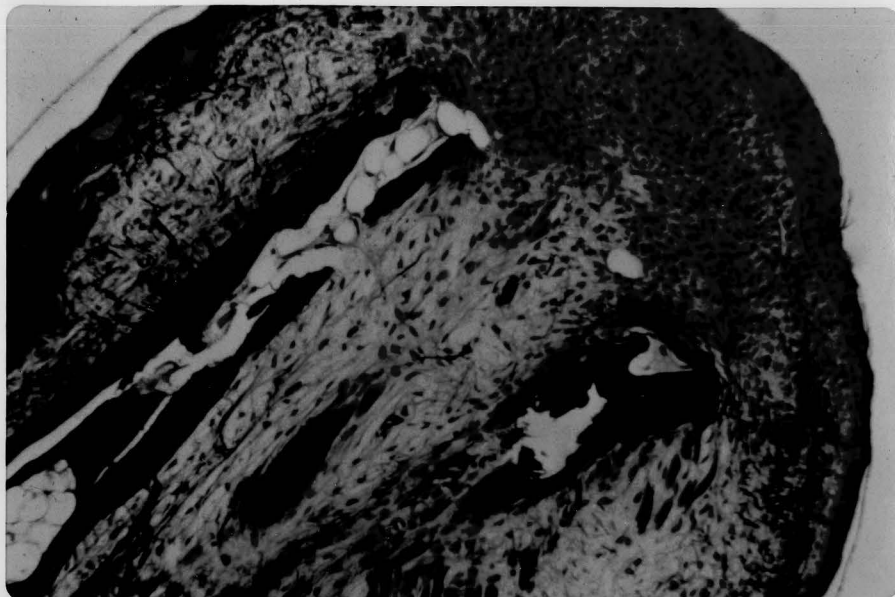


Figure 9

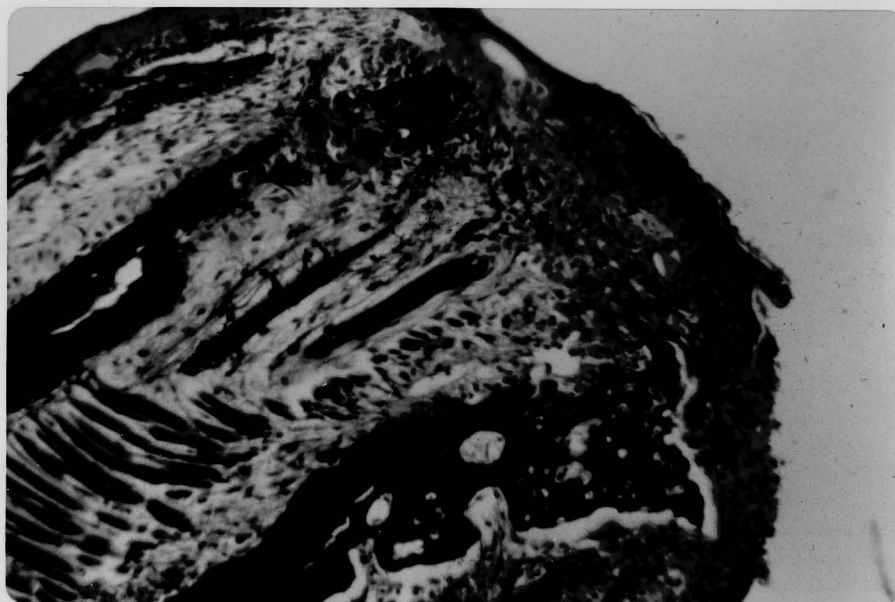


Figure 10

Figure 11: Pseudoblastema of amygdalin-injected
newt (40X).

Figure 12: Spindle-shaped fibrocytes of pseudoblastema
(100X).



Figure 11

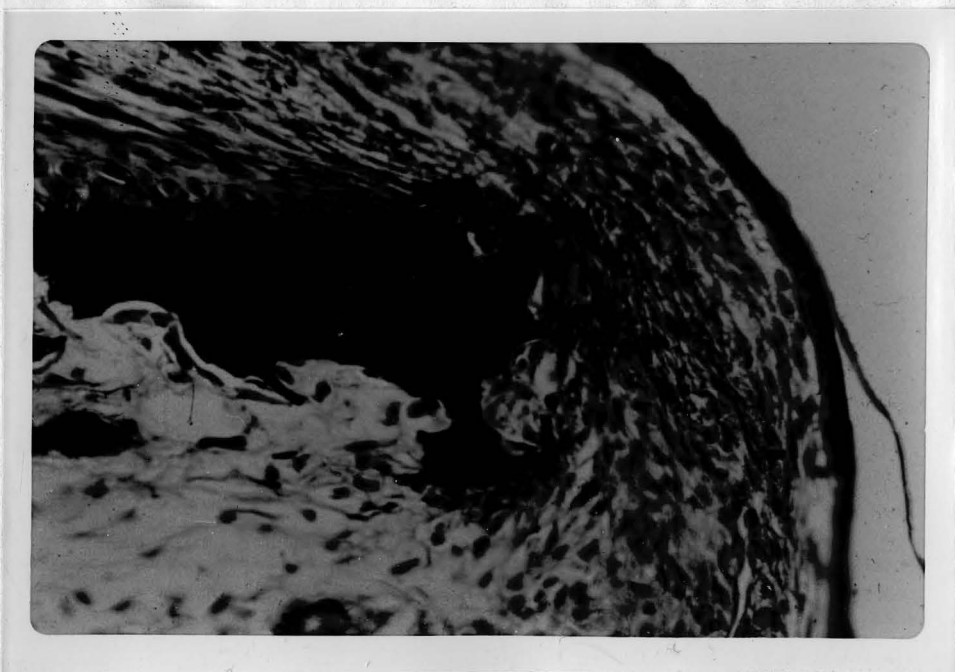


Figure 12

Figure 13: Fibroblasts of control blastema. Note the rounded nucleus and relatively abundant cytoplasm (400X).

Figure 14: Fibrocytes of pseudoblastema. Note attenuated nucleus and sparse cytoplasm (400X).

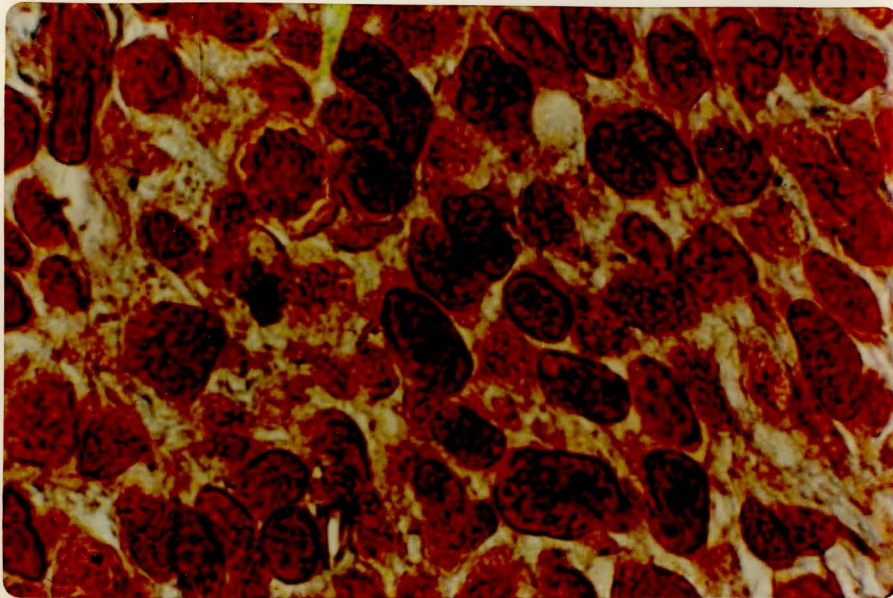


Figure 13

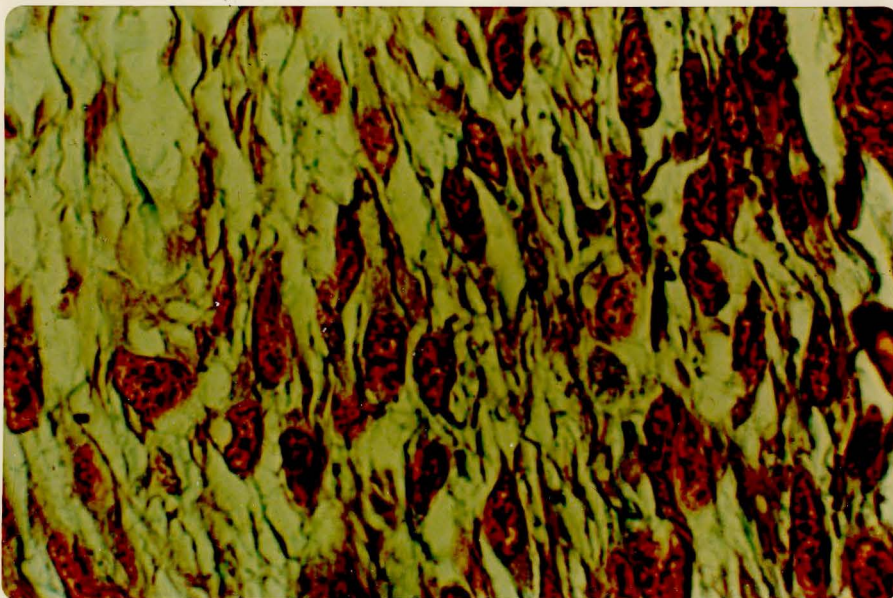


Figure 14

Figure 15: Amygdalin-injected limb (25X).

Figure 16: Edema was prevalent in the amygdalin-injected groups (25X).



Figure 15

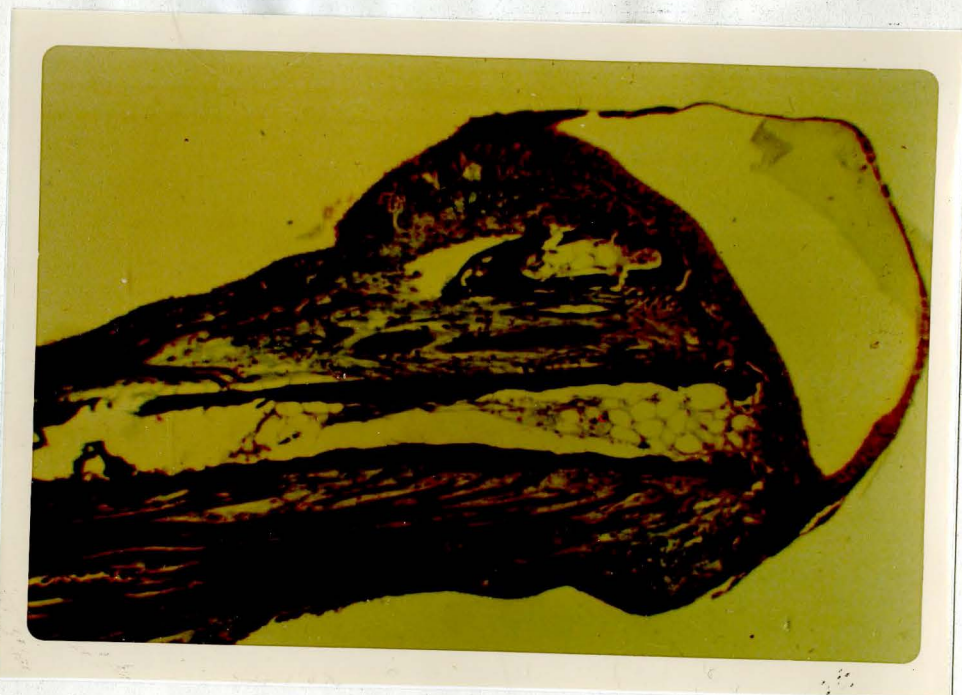


Figure 16

Figure 17: Thickness of the wound epidermis and its underlying basement membrane was similar in both control and experimental groups (400X).

Figure 18: Polymorphonuclear leukocytes were present in the connective tissue of both groups (400X).

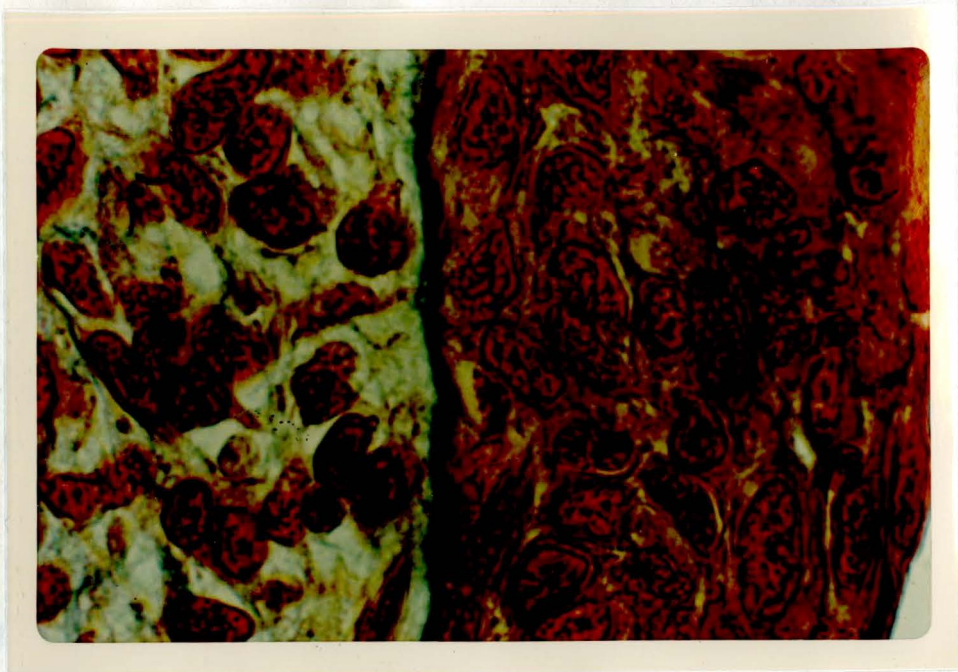


Figure 17

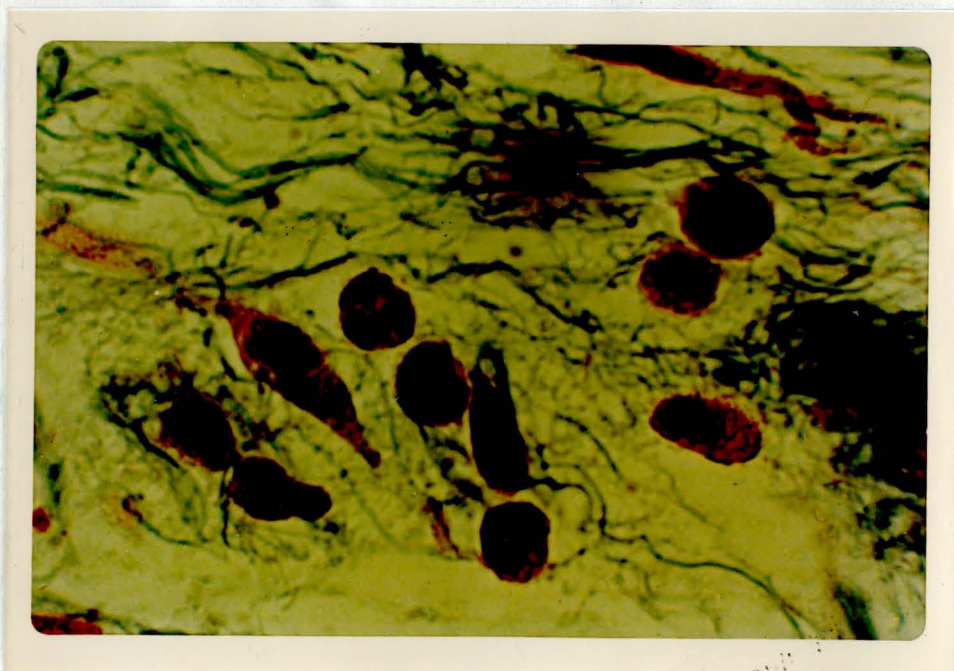


Figure 18

Figure 19: Cartilage appeared similar in both groups (200X).

Figure 20: Muscle appeared similar in both groups (400X).

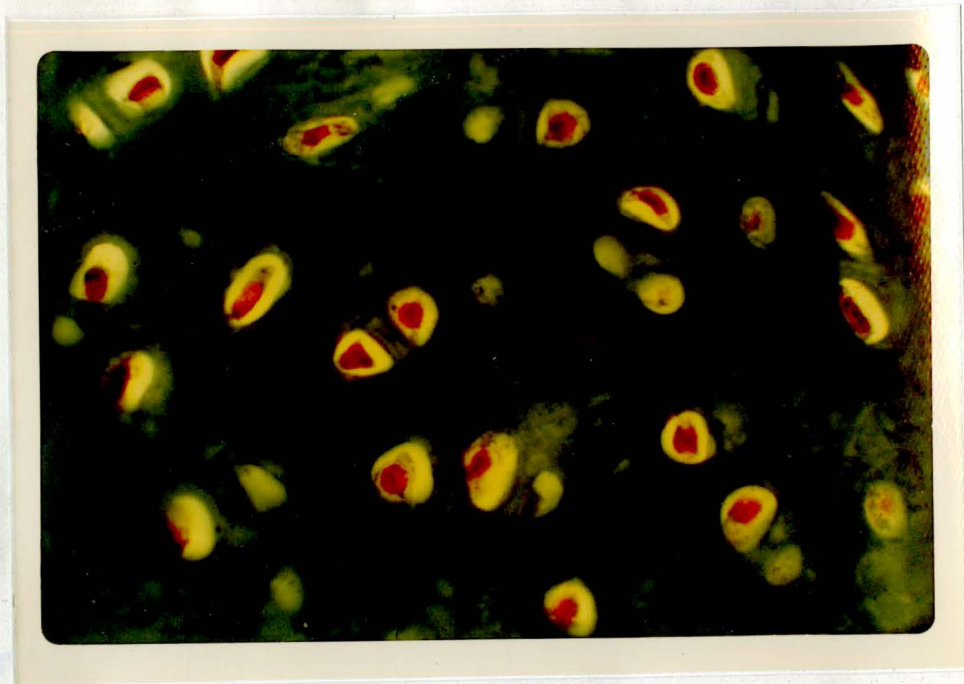


Figure 19

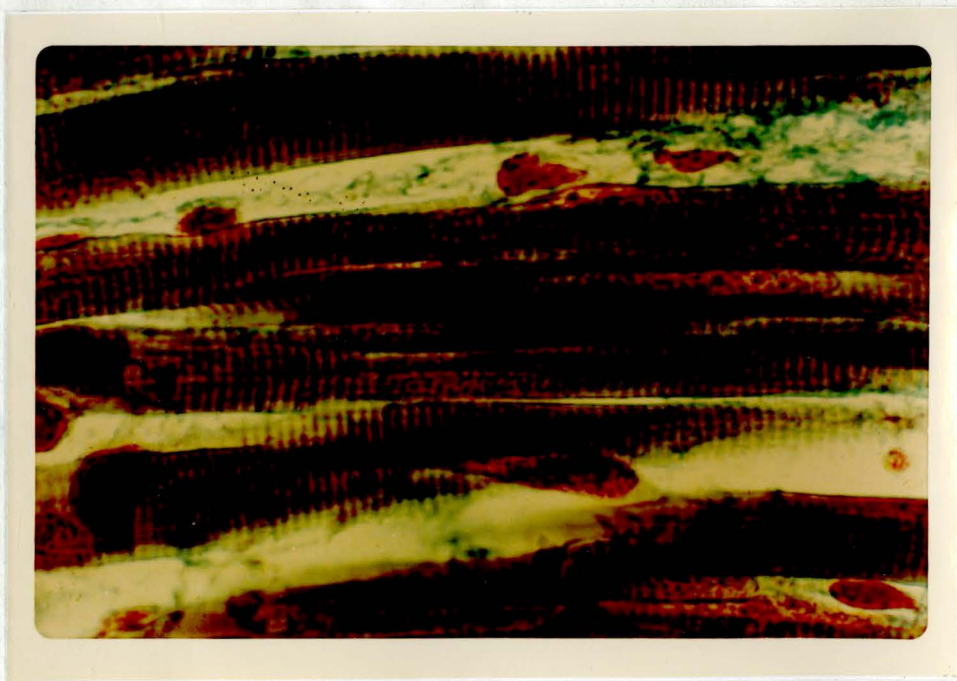


Figure 20

Figure 21: Bone appeared normal in both control and experimental groups (200X).

Figure 22: Osteoclasts, an indication of bone resorption, were present in both groups. These osteoclasts contain debris in their cytoplasm (400X).



Figure 21

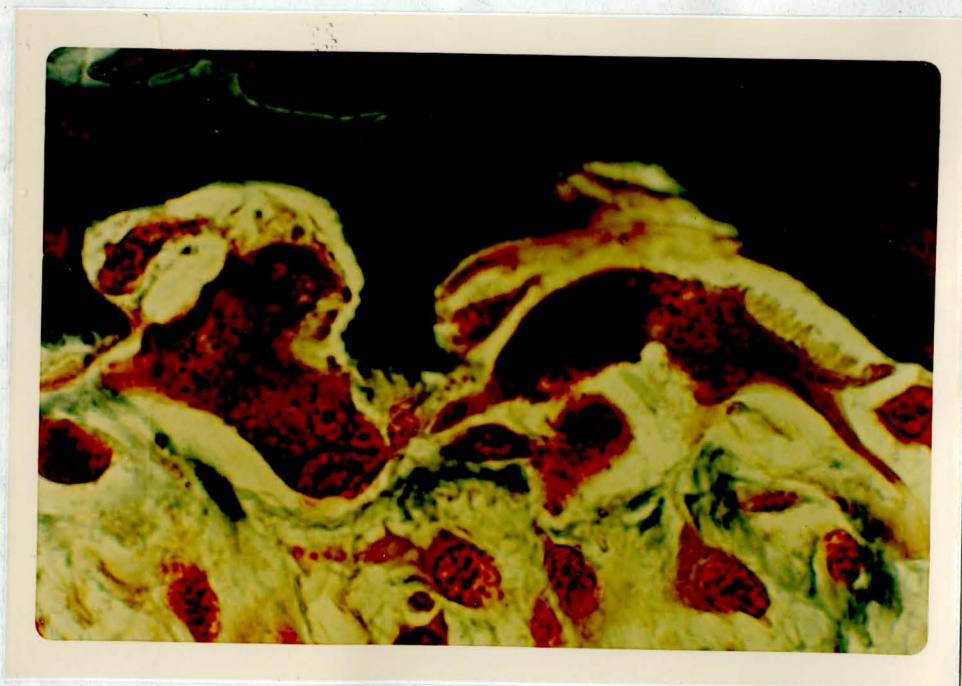


Figure 22

DISCUSSION

Immediately after amputation of the newt limb, there is a sudden decrease in epidermal mitotic activity, which is probably caused by the systemic shock inflicted upon the organism (Manner, 1953). In normal regeneration, blood clots at the wound surface within a few minutes after limb amputation, and basal cell migration from the peripheral epidermis forms an epithelium over the wound. This depletes the normal number of epidermal cells of the stump, and the subsequent increase in mitotic activity of the peripheral stump tissues compensates for this.

Basal cells (which are the least differentiated of epidermal cells) adjacent to a wound migrate over the underlying connective tissue as a single sheet of contiguous cells, which gradually increases in thickness as more cells move in. Migrating cells usually do not divide. Mitosis and synthesis of DNA occurs in the epidermal wound margin (Hay, from Greep, 1966).

The adepidermal membrane is a continuous submicroscopic membrane under the basal cell layer of the epidermis which separates the epidermis from the microscopic basement membrane. In early regeneration, there is a loss of the adepidermal reticulum of fibers, so subjacent cells are able to make contact with the basal cells of the

wound epithelium. Thus, no structural barrier exists between the basal epidermal cells and mesenchymal blastema cells. The adepidermal membrane forms preceeding or simultaneously with formation of the basement membrane (Salpeter and Singer, 1960).

Without epidermis, regeneration would be impossible. If the limb is stripped of skin, the bare parts become covered with epidermis. Healing can be prevented by grafting these skinless limbs to an internal location of the body, e.g., coelomic cavity. Regeneration will not occur until the limb is brought out into the exterior, to permit epidermis to cover the stump. Epidermis transplanted from most other parts of the body onto the stump usually permits regeneration, so the original type of epidermis is not necessary for regeneration (Goss, 1969).

It has been shown that regeneration was inhibited if the full thickness of the skin was ligated over the amputated limb. This proved that dermis must not cover the healing stump in order to allow the wound epithelium to make contact with the underlying mesodermal tissues. There seems to be an interaction between ectoderm and mesoderm in the developing limb bud, although practically nothing is known about the chemical basis of this interaction. Thornton's experiments on the apical epidermal cap conclusively proved that the blastema develops only under the influence of the wound epidermis. If the apical cap is

experimentally removed daily, the production of the blastema is delayed or inhibited, depending on how fast a new apical cap is formed (Thornton, 1954; 1957; 1958; 1965).

Hay and Fishman (1961) showed that the origin of cells of the blastemata proper is from muscles, connective tissue, cartilage, perichondrium, periosteum, perineurium, and dermis. These cells dedifferentiate (from the second to fifth day onward) and morphologically lose their identities as they transform into the fibroblast of the blastema. Thus, specialized cells are all reduced to a common denominator (e.g., in muscle, there is a dissolution of myofibrils so there is nothing left in the cytoplasm but the organelles common to most cells).

Why doesn't each tissue directly give rise to its counterpart and remain recognizable? Goss (1969) considers two possibilities: first, to facilitate mitosis; second, to enhance pluripotentiality, or what Manner (1953) also called its embryonic properties. It is not known whether these cells reconvert to what their previous existence was.

Beginning on about the sixth day, the connective tissue and its cells migrate between the stump and its overlying epidermis. Manner (1953) called this "the stage of the origin of the regeneration blastema." Mesenchymal mitotic activity of fibroblasts increases and the

epidermal mitotic activity declines. After this stage, there is a predominance of fibroblasts in the blastema. Very little transformation of tissues that are located outside of the immediate wound vicinity occurs.

On the 16 postamputational day, both the epidermal and the mesenchymal mitotic activity increase to reach their maximum (Manner, 1953). The maximum epidermal thickness has been reported to be from eight cells (Norman and Schmidt, 1967) to seventeen cells (Manner, 1953) thick. At this time, there is also an increase in the amount of connective tissue making up the blastema.

Normal regeneration did occur in the control group of this experiment. Epidermal thickness was similar in both the control and amygdalin-injected groups. Blastema regeneration, however, was inhibited by amygdalin. Many experimental (amygdalin-injected) animals had no blastema at all. In the experimental animals that did form a "blastema", there was an obvious difference in cell content. Instead of the blastema being composed of mainly fibroblasts, there was a predominance of fibrocytes in the blastema.

Fibroblasts have a large rounded or slightly oval nucleus. They have relatively abundant cytoplasm and are strongly basophilic. Fibrocytes are fusiform or spindle-shaped. The nucleus has one or two nucleoli, with a

small amount of chromatin. Mitochondria are relatively sparse. Cytoplasm is scant and stains weakly basophilic.

Fibroblasts are found abundantly in developing or repairing tissue. They are active in proliferation and in the production of collagen and other connective tissue components. Generally, they are active in protein synthesis and secretion (the endoplasmic reticulum with ribosomes is abundant in the cytoplasm; the Golgi apparatus is also prominent). Fibroblasts also synthesize mucopolysaccharide for ground substance.

Fibrocytes normally inhabit fully differentiated tissue. They are regarded as inactive, fixed cells. They are probably the form adapted by the fibroblasts after they have completed the period of active synthesis. They have less mitochondria, less endoplasmic reticulum, an insignificant Golgi apparatus, and pronounced chromatin (in contrast to the thin chromatin layer of the fibroblasts). Their scant cytoplasm is weakly basophilic.

Although fibrocytes change position little, they retain throughout adult life a capacity for regeneration. When stimulated to proliferate, they are able to make small, gliding movements (Porter, from Greep, 1966).

The growth and the component cells of the blastema seem to be the only factors affected by the amygdalin. Of the amygdalin-injected newts, there was either

no blastema growth, or, if there was, then the "blastema" was a pseudoblastema, composed predominantly of fibrocytes instead of fibroblasts. Edema was prevalent in the amygdalin-injected newts.

Growth of the blastema is postulated to be inhibited by the amygdalin. It is possible that this inhibition is due to the effect of cyanide liberated from the amygdalin molecule. The newt blastema contains appreciable concentrations of the enzyme β -glucuronidase (Stolk, 1962; Dukiet and Niwelinski, 1960), which has generally been found to be in high concentrations in tumors and proliferating cells (Levy, Kerr, and Campbell, 1948; Fishman, Baker, and Borges, 1959). β -glucuronidase concentration is often used as an index of growth (Kerr, Campbell, and Levy, 1948). The occurrence of β -glucuronidase is paralleled by high concentrations of β -glucosidase (Culbert, 1974), which breaks down amygdalin.

If the blastema is fairly high in β -glucosidase and low in rhodanese, there will not be enough rhodanese to detoxify the cyanide, and the fibroblasts of the blastema (which have high oxygen requirements due to their active metabolism and proliferation) will be harmed.

A setback to this postulation is that rhodanese is deficient in brain and muscle tissue, and thus cyanide

might also affect these tissues. However, no adverse muscular or ambulatory effects were noticed in the newts of this experiment. Nerve, which is very important in the regeneration process, was not studied in this experiment. Possibly, amygdalin might have had an effect on nervous tissue. The mechanism of action of amygdalin and the enzymes that are postulated to act on this drug are currently being studied.

SUMMARY

Amygdalin has been postulated to be an inhibitor of pluripotential cells. This hypothesis was tested on the fibroblasts involved in blastema formation in regenerating salamander limbs. The right forelimb of each salamander was amputated at the mid-radius-ulna area. The experimental animals were injected daily for 15 days with 2000 mg/kg of amygdalin. The control group received amphibian ringers solution. The limbs were re-amputated for study on the 16th day. Microscopic examination revealed no significant difference in the epidermal thickness of the two groups. The pluripotential fibroblasts, however, were significantly inhibited: the blastema of the experimental group developed to a mean length of only 110.84 micrometers in comparison to the normal mean length of 266.25 micrometers in the control group.

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APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements of the Master of Science degree.

Date

May 9, 1977

Director's Signature

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